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Silencing Essential Protein Secretion in *Mycobacterium smegmatis* by Using Tetracycline Repressors▽

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Many processes that are essential for mycobacterial growth are poorly understood. To facilitate genetic analyses of such processes in mycobacteria, we and others have developed regulated expression systems that are repressed by a tetracycline repressor (TetR) and induced with tetracyclines, permitting the construction of conditional mutants of essential genes. A disadvantage of these systems is that tetracyclines function as transcriptional inducers and have to be removed to initiate gene silencing. Recently, reverse TetR mutants were identified that require tetracyclines as corepressors. Here, we report that one of these mutants, TetR r1.7, allows efficient repression of *lacZ* expression in *Mycobacterium smegmatis* in the presence but not the absence of anhydrotetracycline (atc). TetR and TetR r1.7 also allowed efficient silencing of the essential *secA1* gene, as demonstrated by inhibition of the growth of a conditional mutant and dose-dependent depletion of the SecA1 protein after the removal or addition, respectively, of atc. The kinetics of SecA1 depletion were similar with TetR and TetR r1.7. To test whether silencing of *secA1* could help identify substrates of the general secretion pathway, we analyzed the main porin of *M. smegmatis*, MspA. This showed that the amount of cell envelope-associated MspA decreased more than 90-fold after *secA1* silencing. We thus demonstrated that TetR r1.7 allows the construction of conditional mycobacterial mutants in which the expression of an essential gene can be efficiently silenced by the addition of atc and that gene silencing permits the identification of candidate substrates of mycobacterial secretion systems.

The genus *Mycobacterium* consists of more than 100 species found in soil or water or in association with plants, animals, or humans (34, 44). Many of the mycobacteria found in association with humans cause disease, and some are among the most prominent human pathogens, including members of the *Mycobacterium tuberculosis* complex and the leprosy bacillus *Mycobacterium leprae*. Diseases caused by these pathogens remain a major global public health problem. For example, one-third of the world population is estimated to be infected with *M. tuberculosis* and each year approximately 9 million people develop the active and infectious form of tuberculosis (TB) (1, 14). Control of TB depends almost entirely on chemotherapy, because efficient vaccines preventing infection or the progression of disease in adults are not available. Successful chemotherapy of TB requires continuous treatment with multiple drugs for at least 6 months and is further complicated by the spread of *M. tuberculosis* strains that are resistant to several or all first-line TB drugs (22). Active disease caused by drug-resistant *M. tuberculosis* is therefore difficult to treat and associated with high rates of mortality. New drugs against TB are thus needed to prevent further deterioration of global public health control due to mycobacterial infections.

Many bacterial virulence factors that facilitate host cell attachment and entry or manipulation of the host immune system are extracytoplasmic proteins (12), and secreted proteins have been crucial to the development of vaccines against diseases caused by bacterial pathogens (20). In addition, protein secretion is also essential for the growth of many bacteria, both in vitro and during infections. Protein export pathways have therefore been extensively characterized in many bacterial pathogens. More recently, the importance of protein secretion systems, e.g., the ESAT-6 (Esx-1) (4, 5, 13, 15–17, 26, 42), the twin-arginine translocation (Tat) (27, 33, 36), and the general secretion (Sec) systems (2, 3, 23), for the growth and virulence of mycobacteria has been analyzed. The Esx-1 system seems to be a specialized transport system that mediates the export of some of the main *M. tuberculosis* antigens, whereas the Tat and Sec systems are important for the extracytoplasmic localization of many proteins. The Esx-1 system is important for virulence but dispensable for the growth of *M. tuberculosis* outside of the host. In contrast, components of the Tat and Sec systems are likely involved in many cellular functions and essential for the growth of *M. tuberculosis* in vitro, which complicates genetic analyses of these systems during infections.

The Sec pathway, also referred to as the general secretion

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
M. smegmatis mc^{2}155	Electroporation-proficient mutant of *M. smegmatis* mc^{2}6	40
MSE10	Hyg^r mc^{2}155 derivative in which *secA1* is controlled by P_{myc1}*tetO*	This work
MSE11	Kan^r mc^{2}155 derivative containing *tetR* integrated into L5 *attB*	This work
MSE12	Kan^r mc^{2}155 derivative containing pTEK-4S0X expressing *tetR* r1.7	This work
MSE13	Kan^r MSE10 derivative containing *tetR* integrated into L5 *attB*	This work
MSE14	Kan^r MSE10 derivative containing pTEK-4S0X expressing *tetR* r1.7	This work
Plasmids		
pKIsecA1	pSE100 derivative in which the mycobacterial origin of replication was replaced by the first 641 bp of *secA1*	This work
pMC1s	Derivative of pMV306K with *tetR* under the control of P_{smyc}	10
pME0L1	Derivative of pMS2 with *lacZ* under the control of P_{myc1}*tetO*	10
pME1mL1	Derivative of pMS2 with *lacZ* under the control of P_{myc1}*tetO* and *tetR* under the control of P_{smyc}	10
pMS2	Hyg^r; a shuttle vector containing origins of replication for *E. coli* (pMB1) and mycobacteria (pAL500) and a multiple cloning site flanked by two transcriptional terminators	19
pMV306K	Kan^r; single-copy-integrating plasmid which inserts into L5 *attB*	43
pSE100	pMS2 derivative containing P_{myc1}*tetO*	This work
pTC-0X1L	Derivative of pMV306K containing *lacZ* expressed from P_{myc1}*tetO*	This work
pTE-1M0X	Derivative of pMS2 with *tetR* expressed by P_{imyc}	This work
pTE-1S0X	Derivative of pMS2 with *tetR* expressed by P_{smyc}	This work
pTE-4M0X	Derivative of pMS2 with *tetR* r1.7 expressed by P_{imyc}	This work
pTE-4S0X	Derivative of pMS2 with *tetR* r1.7 expressed by P_{smyc}	This work
pTEK-4S0X	Kan^r; derivative of pTE-4S0X	This work
$pWH1925_{15A/17G/25V}$	*E. coli* plasmid expressing *tetR* r1.7	38

pathway, has been most extensively studied in *Escherichia coli*, where it is responsible for the transport of the majority of extracytoplasmic proteins. The Sec machinery consists of two cytoplasmic proteins, SecA and SecB, and three integral cytoplasmic membrane proteins, SecY, SecE, and SecG. SecYEG associate in a heterotrimeric complex that forms the channel through which unfolded substrate proteins can pass the cytoplasmic membrane (28). Transport through this pore is facilitated by accessory proteins (SecD, SecF, and YajC) (31) and energized by the ATPase SecA (6, 25, 39). In addition to providing the driving force for transport, SecA is also crucial for the recognition and delivery of substrate proteins to the pore (9) and, thus, central to the general secretion pathway.

While *E. coli* contains one *secA* gene, a number of gram-positive bacteria and acid-fast bacilli, including *M. tuberculosis* and the nonpathogenic, relatively fast-growing *Mycobacterium smegmatis*, contain two *secA* homologs, *secA1* and *secA2* (2). SecA2 is an accessory factor of the general secretion system that promotes the transport of some proteins, e.g., superoxide dismutase and catalase peroxidase in *M. tuberculosis* (3). SecA2 is essential for the virulence of *M. tuberculosis* but dispensable for the in vitro growth of *M. smegmatis* and *M. tuberculosis* (2, 3, 23). SecA1 is essential for the in vitro growth of both species. In contrast to the SecA2 substrates, little is known about the proteins transported by SecA1 in mycobacteria, because mutants harboring a *secA1* deletion could not be obtained. To facilitate the analysis of this essential protein transport pathway in mycobacteria, we sought to construct a conditional *secA1* mutant in *M. smegmatis*.

In mycobacteria, conditional mutants have primarily been generated by using regulated expression systems that silence the target gene by transcriptional repression (for a recent review, see reference 11). Five regulated expression systems based on regulatory elements of the *M. smegmatis* acetamidase gene (Ami system), genes involved in plasmid transfer in *Streptomyces nigrifaciens* (Tra system), or genes encoding tetracycline exporters in *E. coli* or *Corynebacterium glutamicum* (Tet systems) have been developed for mycobacteria. The Tra system is regulated by temperature and requires cultivation at 28°C, a temperature at which mycobacteria grow very slowly, to initiate gene silencing. The Ami system and Tet systems are controlled by short aliphatic amides (e.g., acetamide) and tetracyclines, respectively, and thus allow regulation under conditions optimal for mycobacterial growth. However, a disadvantage of both systems for the construction of conditional mutants is that acetamide and tetracycline function as inducers of gene expression and have to be removed to initiate silencing of the gene under investigation. The regulation of the Ami system involves three *trans* factors and, therefore, seems difficult to manipulate. By contrast, the regulation of the three mycobacterial Tet systems is mediated by a single transcriptional repressor of the well-characterized class of tetracycline repressors (TetRs). Recently, mutant TetRs have been identified that permit gene silencing in *E. coli* (38) and *Bacillus subtilis* (18) by the addition instead of removal of tetracyclines. Here, we report that one of these reverse TetRs (revTetRs) allows efficient gene silencing and the construction of a conditional *secA1* mutant in *M. smegmatis*.

MATERIALS AND METHODS

Bacterial strains, media, reagents, and molecular biology techniques. The bacteria and plasmids used in this study are listed in Table 1. The plasmids were generated by using standard cloning procedures. The correct sequence of all cloned PCR fragments was confirmed by DNA sequencing. The cloning strategies and sequences of the oligonucleotides used for cloning of the different plasmids are available upon request. *Mycobacterium smegmatis* was grown at 37°C on Difco 7H11 agar (BD) or in Difco Middlebrook 7H9 broth (BD) with 0.2% (vol/vol) glycerol and 0.05% (vol/vol) Tween 80. Antibiotics were added, where appropriate, at 50 μg/ml (hygromycin B; Calbiochem) and 15 μg/ml

(kanamycin A; Sigma). Anhydrotetracycline (atc) was obtained from Sigma. The preparation of electrocompetent cells, conduction of electroporation, and preparation of the genomic DNA of *M. smegmatis* were performed as described previously (10).

β-Galactosidase assays. The levels of repression and induction with atc were determined after the cotransformation of *M. smegmatis* mc^{2}155 with two plasmids, one that expressed wild-type (wt) TetR or the revTetR (TetR r1.7) and another that contained *lacZ* downstream of the TetR-responsive promoter P_{myc1}*tetO*. From each transformation, three single colonies were inoculated separately into 2 ml of 7H9 medium containing kanamycin and hygromycin (TetR r1.7). After shaking at 37°C for 44 to 48 h, the bacteria were diluted 100-fold into 1 ml fresh media in 96-square-well plates (Beckman). After shaking at 37°C for 20 to 24 h, the optical densities at 580 nm (OD_{580}) were measured. In a black 96-well plate, 100 μl bacteria were then mixed with 10 μl of 330 μM 5-acetylaminofluorescein di-β-D-galactopyranoside (Invitrogen) (35). The plate was incubated in the dark for 3 h at 30°C and the fluorescence was measured at 515 nm after excitation at 485 nm using a Spectramax M2 plate reader (Molecular Devices). The fluorescence intensities were normalized to the cell densities and reported as relative fluorescence units. All measurements were repeated at least twice.

Replacement of the native *secA1* promoter with P_{myc1}*tetO*. The suicide plasmid pKIsecA1 was generated by amplifying the first 641 bp of *secA1* by PCR, restriction of the amplicon with SphI and NotI, and ligation into the equally restricted pSE100. pKIsecA1 was electroporated into *M. smegmatis* mc^{2}155, and transformants were selected on 7H11 agar plates supplemented with hygromycin B. The site-specific integration of pKIsecA1 was confirmed by PCR using primers P117 (5′-AAGCGGAAGAGCGCCCAATAC-3′) and P120 (5′-CCGCGGAAATAT TCGCCACTTAC-3′), as well as primers P154 (5′-GTCTTTGACGGCTGACA GAG-3′) and P155 (5′-GCGTTGTTCAGGTAGCTGAC-3′).

Culture conditions for *tetR*-containing *secA1* mutants. Mutants containing wt TetR were maintained in the presence of 10 ng/ml atc to allow the expression of *secA1*. Transcriptional silencing of *secA1* was initiated by washing cells grown to a density of OD_{580} of ~2 absorbance units per cm [AU/cm] three times with 7H9 medium free of atc and diluting these bacteria into 7H9 medium without atc. Mutants containing revTetR were maintained in the absence of atc; to silence *secA1*, atc was added to new cultures at the concentrations indicated. To avoid the inactivation of atc, cultures containing atc were protected from light by wrapping the culture vessels with aluminum foil.

Preparation of cell lysates and subcellular fractions. *M. smegmatis* was harvested by centrifugation and washed with phosphate-buffered saline (128 mM NaCl, 8.5 mM Na_2HPO_4, 3.5 mM KCl, 1.5 mM KH_2PO_4, pH 7.4). The cells were resuspended at an OD_{580} of ~10 AU/cm in phosphate-buffered saline supplemented with protease inhibitors (Roche Applied Science), and 1 ml of the suspension was transferred into a screw-cap vial containing approximately 350 μl of 0.1-mm zirconia/silica beads (BioSpec). The cells were broken by shaking in a Precellys 24 homogenizer (Bertin Technologies) at 6,500 rpm for 20 s three times, with 5-min incubations on ice in between. Total lysates were obtained after removing beads and unbroken cells by centrifugation at 8,000 × *g* for 20 s. For detection of MspA, total lysates were centrifuged at 20,800 × *g* for 45 min at 4°C in an Eppendorf 5417R centrifuge to separate the soluble (cytoplasm) and insoluble (cell wall) fractions.

Anti-SecA1 and anti-SecA2 antibody production. Antipeptide antibodies for each SecA in mycobacteria (SecA1 or SecA2) were prepared by using peptides specific for each of the proteins. The peptides were synthesized, conjugated to keyhole limpet hemocyanin, and used to immunize New Zealand White rabbits at Quality Controlled Biochemicals (Hopkinton, MA). For anti-SecA1 antibodies, the peptide RLRERGLDPVETPEEYEA was used (this peptide sequence is identical in *M. tuberculosis* and *M. smegmatis* SecA1). For anti-SecA2 antibodies, the peptide LDHEPGLDLSKLARPT was used (this peptide was designed from *M. tuberculosis* SecA2, and the corresponding region in *M. smegmatis* SecA2 has four amino acid substitutions). The antibodies were purified by affinity chromatography.

SDS-PAGE and quantitative Western blot analysis. Cell lysates were mixed with sodium dodecyl sulfate (SDS) sample buffer and incubated in a boiling water bath for 5 min for analyses of the levels of TetR or SecA1 and incubated for 15 min for analyses of the levels of MspA. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to a Protran BA83 nitrocellulose membrane (Whatman) at 0°C with a constant voltage of 120 V in transfer buffer (192 mM glycine, 25 mM Tris, 20% [vol/vol] methanol) for 2 h. Affinity-purified polyclonal rabbit antibodies against SecA1 or SecA2 were used at a 1:10,000 dilution in TBST (150 mM NaCl, 10 mM Tris-Cl, 0.05% [vol/vol] Tween 20, pH 8.0). Polyclonal rabbit antibodies against MspA (29) and AhpC (7) were used at a 1:50,000 dilution and a 1:30,000 dilution, respectively, in TBST. For the quantification of TetR, a mixture of hybridoma supernatant containing monoclonal murine antibodies (32) was used at a 1:100 dilution in TBST. Horseradish peroxidase-linked secondary antibodies against rabbit immunoglobulin G or mouse immunoglobulin G (GE Healthcare) were used at a 1:30,000 dilution in TBST. Immobilon Western horseradish peroxidase substrates (Millipore) and BioMax MR films (Kodak) were used for enhanced chemiluminescence detection. The densitometry of Western blots was performed by scanning films with an Epson perfection 2450 photo scanner and analyzing the resultant 16-bit gray scale TIFF images with Quantity One software (Bio-Rad).

RESULTS

Regulation of β-galactosidase expression by TetR and TetR r1.7 in *M. smegmatis*. To determine whether revTetRs function in mycobacteria, we measured the activity of TetR r1.7, one of the most efficient revTetRs, which contains the mutations E15A, L17G, and L25V (38), in *Mycobacterium smegmatis* by using a β-galactosidase reporter assay. For this assay, the TetR-controlled *lacZ* gene was integrated into the *M. smegmatis* chromosome and TetR and TetR r1.7 were expressed episomally (Fig. 1A). The repression of β-galactosidase activities was measured in strains expressing TetR or TetR r1.7 by using promoters with high (P_{smyc}) or lower (P_{imyc}) activities (19). Initially, the bacteria were either grown without atc or with 30 ng/ml atc (Fig. 1B). The expression of wt TetR using either P_{imyc} or P_{smyc} resulted in efficient repression of *lacZ*, but the induction of β-galactosidase with atc was detected only when TetR was expressed using P_{imyc}. The repression of *lacZ* by TetR r1.7 occurred only in the presence of atc and only in bacteria containing TetR r1.7 under the control of P_{smyc}. The maximal regulation factors were 39 for wt TetR and 17 for TetR r1.7. Next, we measured β-galactosidase activities after the cultivation of *M. smegmatis* with atc concentrations ranging from 1 to 200 ng/ml (Fig. 1C). These experiments confirmed that the regulation of *lacZ* by atc was efficient only when wt TetR was expressed by P_{imyc} and TetR r1.7 by P_{smyc}. In both strains, the regulation was atc dose responsive. Induction by TetR reached saturation at 10 ng/ml atc and repression by TetR r1.7 was maximal with as little as 10 ng/ml atc. These experiments demonstrated that TetR r1.7 allows efficient, atc dose-dependent regulation of gene expression in *M. smegmatis* if expressed by a strong mycobacterial promoter.

Expression of TetR and TetR r1.7 in *M. smegmatis*. To test whether the requirement for a strong promoter for TetR r1.7-mediated repression was caused by small in vivo protein amounts, we compared the steady-state levels of TetR and TetR r1.7. Quantitative Western blots were performed using protein lysates from log-phase cultures of *M. smegmatis* transformed with pTE-1S0X or pTE-4S0X (OD_{580} at 1.2 to 1.25 AU/cm). The TetR levels were quantified densitometrically, using AhpC, the *M. smegmatis* alkylhydroperoxidase, for normalization, and known amounts of purified TetRs for quantification. This revealed that each mg of total lysate contained 7.4 ± 2.4 ng TetR but only 1.5 ± 0.9 ng TetR r1.7 (Fig. 2). The amounts of TetR and TetR r1.7 in *M. smegmatis* expressing the respective repressors using P_{imyc} were both below the limit of detection (data not shown). The Western blot analyses thus demonstrated that the steady-state level of TetR r1.7 was approximately one-fifth of that of wt TetR when expression was driven by P_{smyc} in otherwise identical *M. smegmatis* strains.

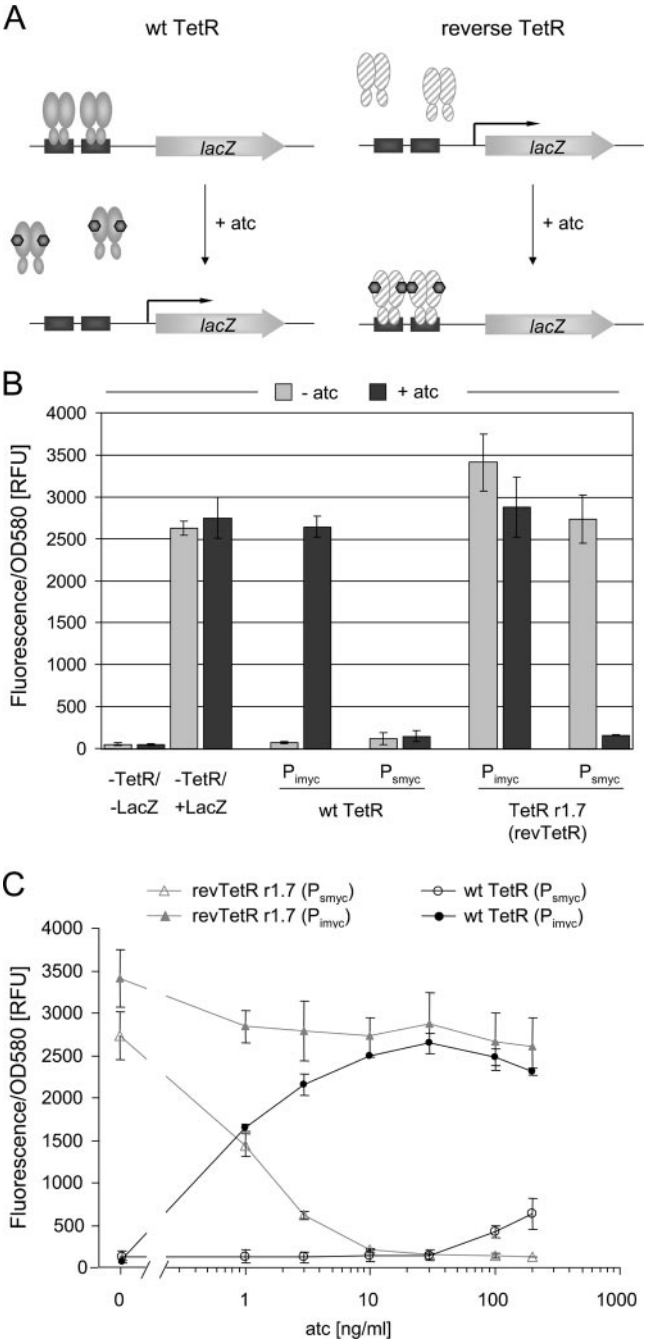

FIG. 1. Regulation of β-galactosidase expression in *M. smegmatis* by wt TetR and revTetR. (A) Schematic of the assay system. wt TetR is depicted by gray ovals and revTetR by striped ovals. The *tetO* operator sites that bind the repressors are shown as black boxes in the TetR-responsive promoter $P_{myc1}tetO$. β-Galactosidase was expressed from a *lacZ* gene located downstream of $P_{myc1}tetO$ and integrated into the *M. smegmatis* chromosome at the mycobacteriophage L5 *att* site. Expression of wt TetR and TetR r1.7 was achieved using episomal plasmids containing the respective *tetR* genes downstream of a strong (P_{smyc}) or weaker (P_{imyc}) promoter. For wt TetR, the expression of *lacZ* is switched on after the addition of atc, depicted by dark gray hexagons (left side). For revTetR, the addition of atc mediates repression of *lacZ* transcription (right side). (B) Repression and induction with TetR, TetR r1.7, and atc. β-Galactosidase activities in relative fluorescence units (RFU) were measured in the absence (−) or presence (+) of 30 ng/ml atc as described in Materials and Methods. The light gray bars represent β-galactosidase activities in the absence of atc, whereas the dark gray bars show β-galactosidase activities after the addition of atc. Promoters P_{smyc} and P_{imyc} were used to achieve high or low expression, respectively, of TetRs. Error bars represent the standard errors of the means. (C) Dose responsiveness of β-galactosidase expression regulated by wt TetR and TetR r1.7 measured in relative fluorescence units (RFU). Regulation mediated by low-level expression of TetR is depicted by filled circles for wt and filled gray triangles for TetR r1.7. Regulation mediated by high-level expression of TetR is shown as open circles for wt and open gray triangles for TetR r1.7. atc concentrations ranging from 1 to 200 ng/ml were used to regulate the transcription of *lacZ*. Error bars represent the standard deviations of the means.

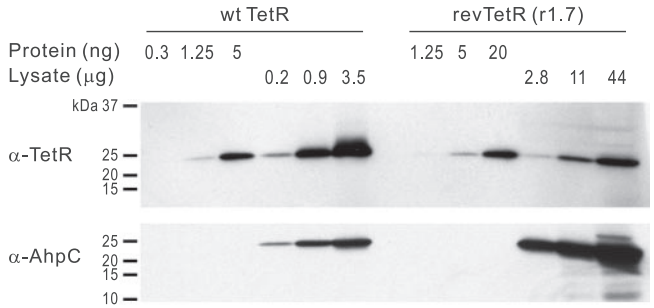

FIG. 2. Steady-state protein levels of TetR and TetR r1.7 in *M. smegmatis*. Total lysates from *M. smegmatis* strains transformed with either pTE-1S0X (expressing wt TetR from the strong promoter P_{smyc}) or pTE-4S0X (expressing TetR r1.7 from the strong promoter P_{smyc}) were loaded on a 10% SDS-PAGE gel in 1:4 serially decreasing amounts for quantitative Western blot analysis using antibodies against TetR (α-TetR). Purified wt TetR and TetR r1.7 proteins were also loaded in 1:4 serially decreasing amounts on the same gel for the calibration of standard curves. Different amounts of wt TetR- and TetR r1.7-containing lysates were used to obtain signal strengths in or near the linear range comparable with that of the purified standards so that quantification could be performed. AhpC was used as the loading control. Positions of the molecular weight markers are indicated on the left.

Construction of conditional *secA1* mutants. SecA1 was shown to be essential for the growth of *M. smegmatis* based on the inability to obtain a *secA1* deletion mutant via allelic exchange (2). To obtain a mutant in which the expression of SecA1 can be controlled by TetRs, we generated the suicide plasmid pKIsecA1 by cloning a PCR product containing a Shine-Dalgarno sequence followed by the first 641 bp of the 5′ coding region of *M. smegmatis secA1* into pSE100 downstream of the TetR-responsive promoter $P_{myc1}tetO$ (Fig. 3A). pKIsecA1 was transformed into *M. smegmatis* mc^2155, and chromosomal DNA was isolated from hygromycin-resistant clones. Analyses by PCR demonstrated that $P_{myc1}tetO$ was inserted into the chromosome upstream of *secA1* (Fig. 3B). In the absence of *tetR*, this mutant, named MSE10, showed normal growth compared to that of *M. smegmatis* mc^2155 (data not shown).

To achieve silencing of *secA1*, MSE10 was transformed with plasmid pMC1s, which integrates a P_{smyc}-*tetR* expression cassette into the mycobacteriophage L5 *att* site, or with the episomal plasmid pTEK-4S0X, which expresses TetR r1.7 using the promoter P_{smyc}, generating strains MSE13 and MSE14, respectively (Fig. 3A). As controls, strains MSE11 and MSE12

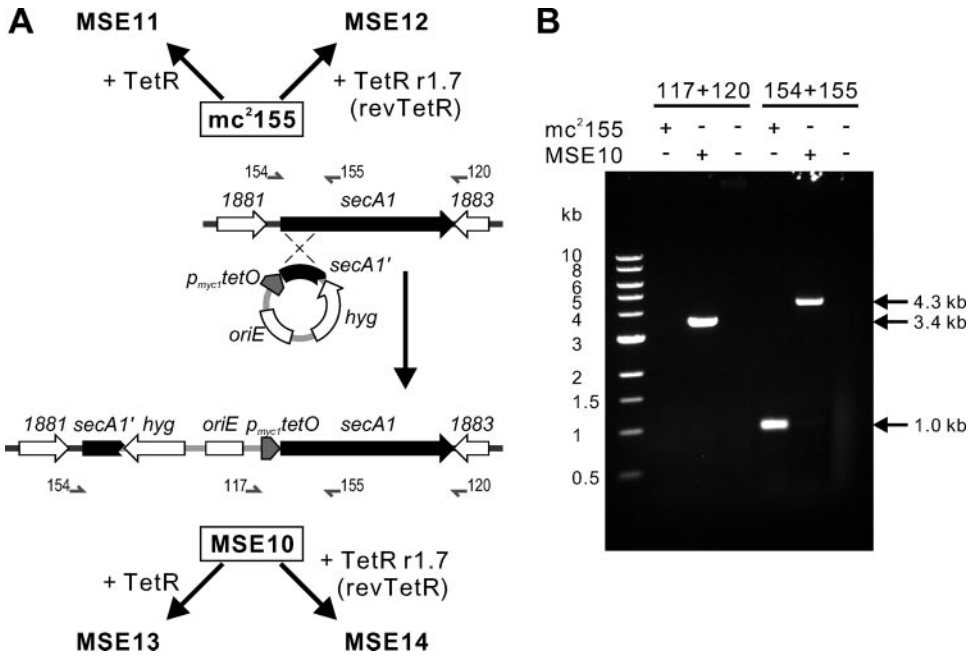

FIG. 3. Construction and verification of conditional *secA1* mutants of *M. smegmatis*. (A) Schematic overview of strain construction. The suicide plasmid pKIsecA1 was electroporated into *M. smegmatis* mc²155 and integrated at the *secA1* locus via homologous recombination, inserting the TetR-responsive promoter P_{myc1}*tetO* before *secA1* and generating strain MSE10. wt TetR or TetR r1.7 was subsequently introduced into MSE10 to generate the conditional *secA1* mutants MSE13 and MSE14, respectively; TetR or TetR r1.7 was also introduced into mc²155 to generate the control strains MSE11 and MSE12, respectively. 1881 and 1883 stand for MSMEG1881 and MSMEG1883, genetic loci upstream and downstream of *secA1* on the genome of *M. smegmatis* mc², according to The Institute for Genomic Research. PCR primers 154, 155, 117, and 120 were used for verifying the genotype of MSE10. (B) Confirmation of the MSE10 genotype by PCR. Genomic DNA prepared from mc²155 or MSE10 was used in PCRs with the indicated primer pairs, and the products were analyzed on a 1% agarose gel. No PCR product was expected using primers 117 plus 120 and the genomic DNA of mc²155; the size of the expected product from primers 117 plus 120 and MSE10 was 3.4 kb. Primers 154 plus 155 were expected to give a 1.0-kb product from mc²155 and a 4.3-kb product from MSE10. The arrows indicate the locations of the products. DNA molecular weight markers are shown on the left.

were generated by transforming *M. smegmatis* mc²155, which contained *secA1* under the control of its native promoter, with pMC1s and pTEK-4S0X, respectively. The four strains were streaked onto 7H11 agar plates containing 50 ng/ml atc or no atc. The control strains grew normally under both conditions, but growth of MSE13 was observed only in the presence of atc, whereas MSE14 grew only in the absence of atc (Fig. 4). This experiment confirmed the essentiality of *secA1* for the growth of *M. smegmatis* on agar plates and demonstrated that efficient growth inhibition was achieved with both wt TetR and TetR r1.7.

Dose dependency of growth inhibition and SecA1 depletion. We next determined whether growth inhibition of strains MSE13 and MSE14 could also be achieved in liquid culture and was atc dose dependent. The mutants were first grown under permissive conditions (i.e., MSE13 was grown in 7H9 medium containing 10 ng/ml atc and MSE14 was grown without atc), washed, and diluted into fresh media containing no atc or 0.03 to 30 ng/ml atc at threefold-increasing concentrations (Fig. 5A). The growth of MSE13 was inhibited in media containing less than 1 ng/ml atc, whereas the growth of MSE14 was impaired in media containing more than 1 ng/ml atc. The extent of growth inhibition was atc concentration dependent in both strains.

The dose dependency of *secA1* silencing was examined by quantitative Western blot analyses measuring SecA1 protein

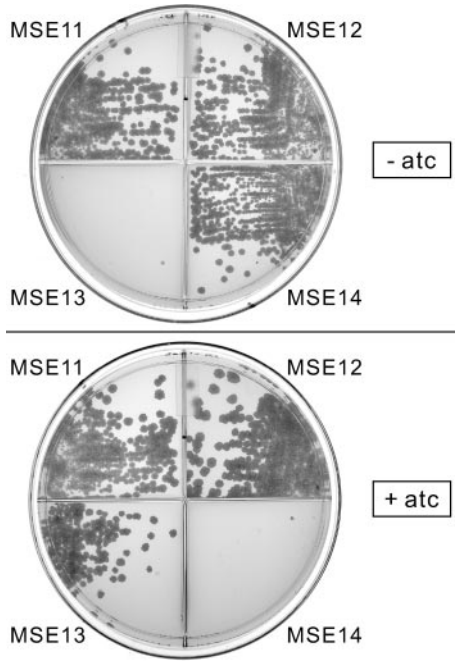

FIG. 4. Growth of *secA1* conditional silencing mutants on agar plates in the absence (−) and presence (+) of atc. wt-TetR-regulated strain MSE13, TetR r1.7-regulated strain MSE14, and the respective control strains MSE11 and MSE12 were streaked onto 7H11 plates without atc or with 50 ng/ml atc, and pictures of the plates were taken after 3 days of incubation at 37°C.

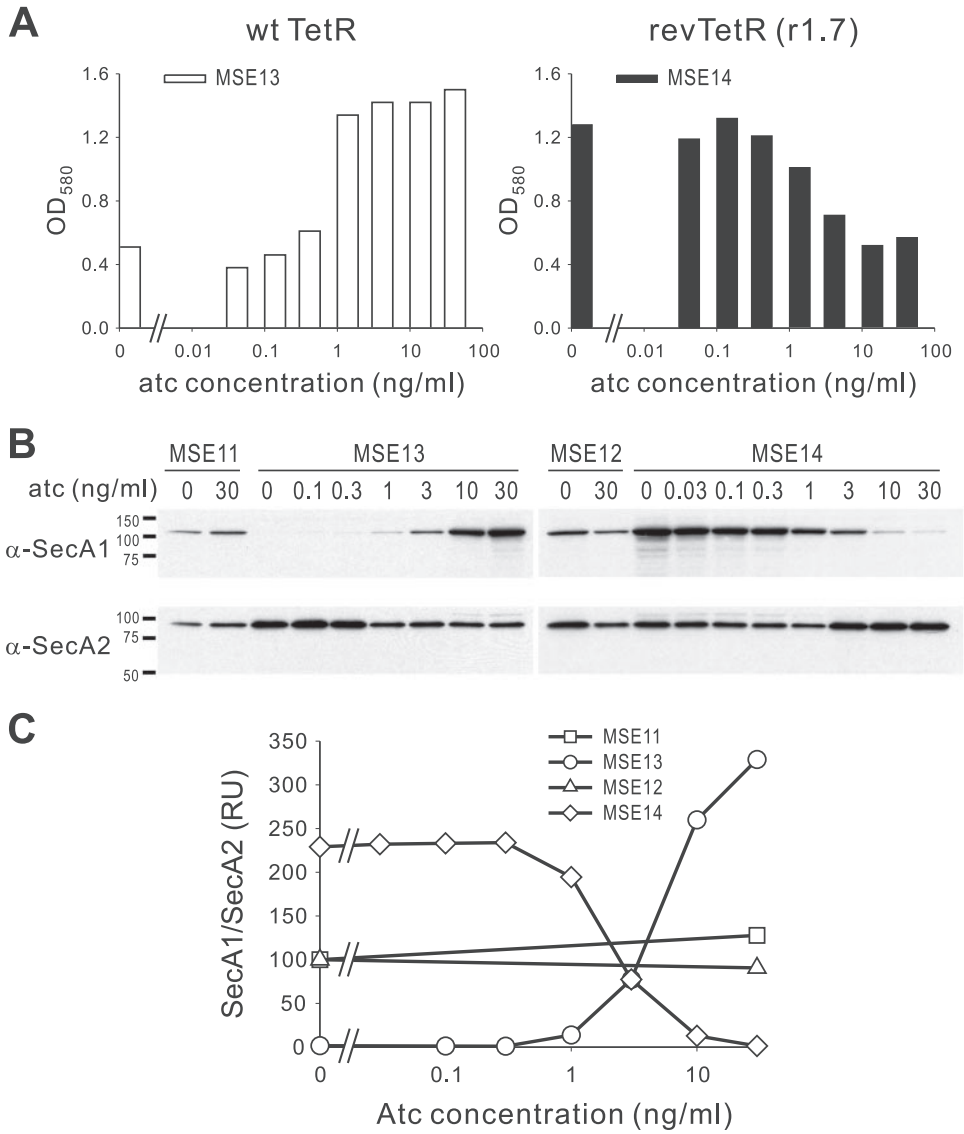

FIG. 5. atc dose dependency of growth inhibition and SecA1 depletion in conditional *secA1* mutants. (A) Growth inhibition. Cultures of *M. smegmatis* strains were initially obtained in media allowing *secA1* expression, washed, and diluted into fresh media containing different atc concentrations at the starting OD_{580} of 0.02 AU/cm. The cultures were agitated at 250 rpm at 37°C for 21 h, and bacterial growth was measured by the OD_{580}. (B) SecA1 Western blot analysis. Total lysates of *M. smegmatis* were prepared from cultures harvested at 21 h from the above experiment and analyzed by 10% SDS-PAGE, followed by Western blotting using antibodies against SecA1 and SecA2 (α-SecA1 and α-SecA2). SecA2 was used as the loading control. Positions of the protein molecular weight markers are indicated on the left. (C) Quantification of the Western blot signals. The signal strengths of the SecA1 and SecA2 bands were determined by densitometry, and the loading-adjusted SecA1 expression levels were calculated by dividing the SecA1 level by the SecA2 level. In order to compare the two groups of strains that were regulated by wt TetR or TetR r1.7 on the same graph, the loading-adjusted SecA1 levels for all samples of MSE11 and MSE13 were expressed as the percentages of the level for MSE11 at 0 ng/ml atc, and the loading-adjusted SecA1 levels for all samples of MSE12 and MSE14 were expressed as the percentages of the level for MSE12 at 0 ng/ml atc. RU, relative units.

levels in bacterial lysates (Fig. 5B and C). The SecA1 levels in control strains MSE11 and MSE12 were not significantly different from that in *M. smegmatis* mc^2155 (data not shown). At atc concentrations of 10 ng/ml or higher, the SecA1 levels in MSE13 were ~300% of that in the control strain MSE11 and a similar increase in SecA1 expression was observed in MSE14 in the absence of atc. In MSE13, the SecA1 levels decreased with decreasing atc concentrations. At 3 ng/ml atc, the SecA1 levels were reduced to 77% of the level in the control, at 1 ng/ml atc to 14%, and at less than 0.3 ng/ml, SecA1 was below the limit of detection. By contrast, in MSE14, the SecA1 levels decreased with increasing atc concentrations. The SecA1 amounts were reduced to 77% at 3 ng/ml atc and 13% at 10 ng/ml and reached the limit of detection at 30 ng/ml. Taken together, these experiments demonstrated that both wt TetR and TetR r1.7 efficiently silenced *secA1* and allowed dose-dependent regulation of SecA1 protein levels over a range of at least two orders of magnitude. The impact of SecA1 depletion on growth confirmed that *secA1* is also essential for the growth of *M. smegmatis* in liquid culture.

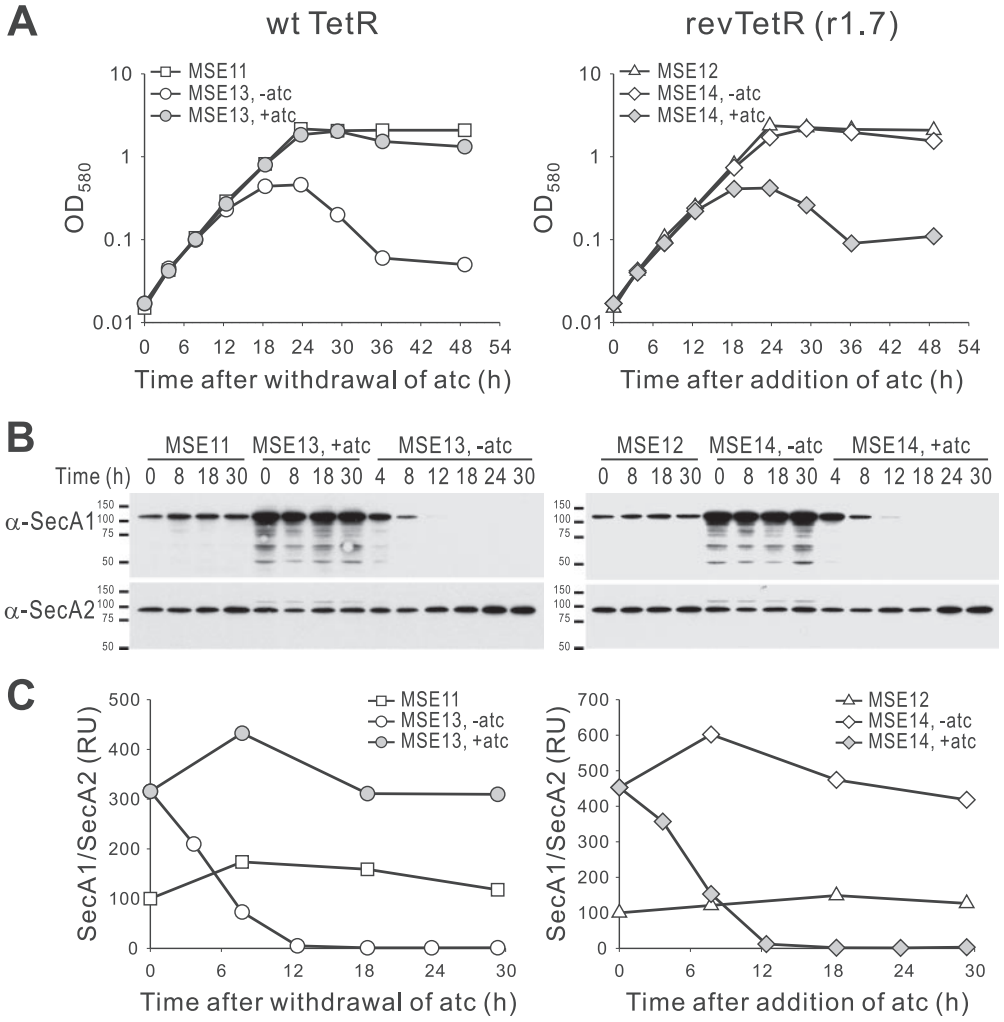

FIG. 6. Kinetics of growth inhibition and SecA1 depletion. (A) Growth inhibition. Cultures of *M. smegmatis* strains were grown in media allowing *secA1* expression, washed, and diluted into fresh media containing no atc (−) or 40 ng/ml atc (+). Bacterial growth was monitored in the following 48 h by measuring the OD_{580}. (B) SecA1 Western blot analysis. Samples of bacterial cultures in the experiment described above were harvested at the times indicated, and total lysates were prepared and analyzed by 10% SDS-PAGE, followed by Western blot analysis using antibodies against SecA1 and SecA2 (α-SecA1 and α-SecA2). SecA2 was used as the loading control. Positions of the protein molecular markers are indicated on the left. (C) Quantification of the Western blot signals, performed as described in the legend to Fig. 5C.

Kinetics of growth inhibition and SecA1 depletion. To determine the kinetics of growth inhibition and SecA1 depletion, strains MSE13 and MSE14 were grown in permissive media, washed, and diluted into new media without or with atc (10 ng/ml for MSE13 and 40 ng/ml for MSE14). Bacterial growth was monitored by OD measurements, and samples of cultures were harvested every 4 h in the early log phase and every 6 h in the mid-log to stationary phases for the detection of SecA1 levels in total lysates by quantitative Western blotting. For both MSE13 and MSE14, the growth rates started to decline approximately 12 h after transfer into nonpermissive media (Fig. 6A); the culture densities peaked at 21 to 24 h and decreased to 0.05 to 0.11 AU/cm in later growth phases. Samples were taken at the indicated time points and analyzed by quantitative Western blotting (Fig. 6B and C). At the start of the time course, the SecA1 level in MSE13 was ~300% of that in the control strain MSE11, and it decreased to ~200% at 4 h after atc removal, 73% at 8 h, and 5% at 12 h and was below the limit of detection at 18 h and the later time points. The SecA1 level in MSE14 was ~450% of that in the control strain MSE12 at the beginning of the experiment, and it decreased to ~350% at 4 h after atc addition, ~150% at 8 h, and 12% at 12 h and was below the limit of detection at 18 h and later time points. The kinetics of growth inhibition and SecA1 depletion were, thus, similar in MSE13 and MSE14.

Impact of *secA1* silencing on steady-state levels of cell wall-associated MspA. In *E. coli*, the transport of porins across the cytoplasmic membranes is *secA* dependent (30). To begin the identification of SecA1 substrates in *M. smegmatis*, we analyzed the impact of *secA1* silencing on the steady-state level of cell wall-associated MspA, the major porin of *M. smegmatis* (41). MSE14 was grown in the same manner as in the dose-dependency experiments in media containing different atc concentrations for 21 h, when the bacterial growth as measured by OD reached a plateau. The cell wall fractions of the bacterial lysates were then examined for MspA by quantitative Western

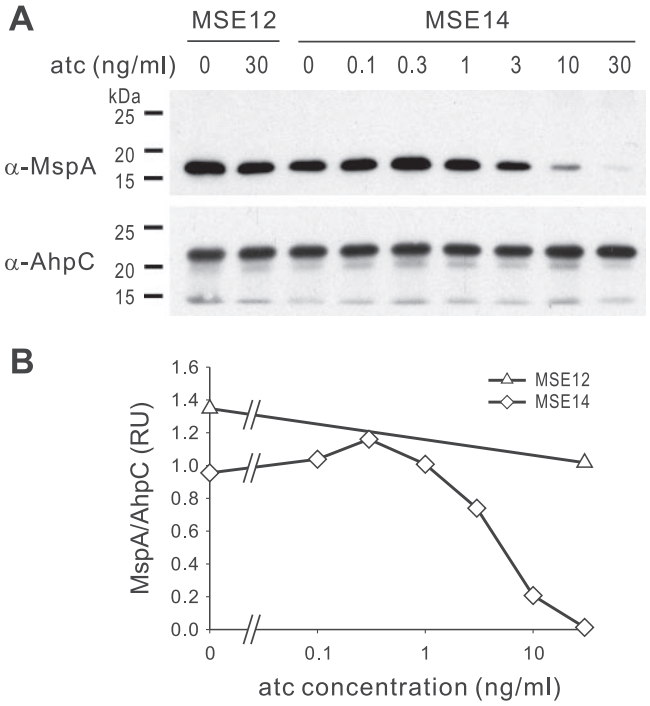

FIG. 7. Impact of *secA1* silencing on steady-state levels of cell wall-associated MspA. (A) MspA Western blot analysis. Cell wall-associated proteins were prepared from MSE12 and MSE14 cultures grown for 21 h with different atc concentrations as described in Materials and Methods and analyzed by 10% SDS-PAGE, followed by Western blot analysis using antibodies against MspA and AhpC (α-MspA and α-AhpC). AhpC was used as the loading control. Positions of the protein molecular markers are indicated on the left. (B) Quantification of Western blot signals. The signal strengths of the MspA and AhpC bands were determined by densitometry, and the loading-adjusted MspA levels were calculated by dividing the MspA level by the AhpC level. RU, relative units.

blotting (Fig. 7). At atc concentrations of 1 ng/ml or lower, i.e., conditions that did not cause a reduction of the SecA1 level compared to the level in the reference strain MSE12 (Fig. 6B and C), the MspA level in the cell wall fraction was almost identical to the level in the control strain (96% to 116%). As the atc concentration was increased to 3 ng/ml or higher, when SecA1 was efficiently silenced, the MspA level in the cell wall fraction decreased to 74% at 3 ng/ml atc, 21% at 10 ng/ml atc, and 1% at 30 ng/ml atc, suggesting that MspA was secreted in an SecA1-dependent manner. MspA was not detected in the cytosolic fraction (data not shown) even when SecA1 was silenced and MspA levels had decreased in the cell wall fraction, suggesting that MspA did not accumulate intracellularly upon *secA1* silencing.

DISCUSSION

Regulated bacterial expression systems have primarily been used for two applications: the production of proteins and the analysis of genes that are essential for bacterial growth. Protein purification is greatly facilitated by expression systems that allow controlled overproduction of the desired protein after the initial propagation of bacteria under conditions that suppress overexpression. The expression systems best suited for the production of proteins are, therefore, systems that can be turned on by the addition of an inducer to a bacterial culture. Such inducible expression systems have also been useful for the analysis of in vivo gene functions (for an example, see reference 45). However, if the silencing of a gene is the goal, such expression systems are not ideal, because the inducer has to be removed to achieve silencing. Removal of the inducer from the bacteria usually requires procedures (e.g., centrifugation and washing) that may affect the biological process under investigation, can be difficult to achieve in some assays (e.g., during oxygen deprivation or infections of eukaryotic cells or animals) and may be slow in some cases due to intracellular accumulation of the inducer or its binding to the bacterial cell envelope. The expression systems best suited for gene silencing are therefore systems that can be turned off by the addition of a corepressor.

RevTetRs, which require tetracyclines as corepressors and allow the repression of gene expression by the addition of tetracyclines to bacterial cultures, have recently been isolated (18, 38). We used *lacZ* reporter gene measurements and a conditional *M. smegmatis secA1* mutant to demonstrate that one of these repressors, TetR r1.7, also allows the silencing of mycobacterial gene expression. Expression of TetR r1.7 by the strong promoter P_{smyc} (19) resulted in a 17-fold-lower β-galactosidase activity in the presence of 30 ng/ml atc than in the absence of atc. The repression of β-galactosidase activity was atc dose dependent and maximal at 10 to 30 ng/ml atc, concentrations that are up to 50-fold lower than the atc concentration of 500 ng/ml that reduced the growth of *M. smegmatis* (10). This demonstrated that TetR r1.7 can be used to efficiently silence gene expression in *M. smegmatis* by the addition of subinhibitory concentrations of atc.

Silencing of β-galactosidase activities by TetR r1.7 was not observed if the repressor was expressed using the weaker promoter P_{imyc} (19). In contrast, repression of β-galactosidase by wt TetR was efficient in strains that contained this repressor under the control of either promoter, and expression of wt TetR by the strong promoter even interfered with efficient induction in response to 200 ng/ml atc. Quantitative Western blot analyses revealed that expression from P_{smyc} led to a fivefold-higher steady-state level of wt TetR compared to that of TetR r1.7, and the steady-state levels of wt TetR and TetR r1.7 expressed from P_{imyc} were both substantially lower than the levels expressed from P_{smyc}. Repression by wt TetR was, thus, efficient at steady-state levels below those that led to repression by TetR r1.7, suggesting that wt TetR is more efficient in *M. smegmatis* than TetR r1.7. The lower efficiency of TetR r1.7 also prevented efficient silencing in strains that expressed this variant chromosomally (data not shown), presumably because multiple gene copies were necessary to achieve sufficient steady-state levels of the repressor required for efficient repression. It is unknown if the lower steady-state level of TetR r1.7 was caused by a lower half-life of the protein in vivo or less-efficient translation of its mRNA. Mycobacterial pAL5000-derived episomes are stable over many generations even in the absence of selection (S. Ehrt, unpublished data). The requirement for episomal expression might, therefore, not significantly limit the use of TetR r1.7 in mycobacteria.

We next constructed an *M. smegmatis* mutant in which transcription of the essential *secA1* was brought under the control

of $P_{myc1}tetO$ and used this mutant to test whether TetR r1.7 permits conditional silencing of *secA1*. The addition of atc inhibited the growth of this mutant both on agar plates and in liquid culture, and the growth inhibition in liquid culture was atc dose dependent. In contrast, the growth of the $P_{myc1}tetO$-*secA1* mutant containing wt TetR required atc both on agar plates and in liquid culture. The kinetics of SecA1 depletion were similar after *secA1* silencing with wt TetR and TetR r1.7. These experiments demonstrate that both wt TetR and TetR r1.7 permit the construction of mycobacterial mutants in which the function of essential genes can be studied by conditional gene silencing. They furthermore confirm the essentiality of *secA1* for the growth of *M. smegmatis* on agar plates and demonstrate that *secA1* is essential for growth in liquid culture as well.

The general secretion pathway has been extensively studied in *E. coli*, but relatively little is known about its function in mycobacteria, even though this protein secretion pathway is likely important not only for in vitro growth but also for the virulence of mycobacterial pathogens (3, 23). To test whether the identification of substrates of the general secretion pathway could be facilitated by conditional silencing of *secA1*, we measured the amount of cell wall-associated MspA before and after *secA1* silencing. A >90-fold, atc dose-dependent reduction of cell wall-associated MspA was detected after silencing *secA1* with TetR r1.7 and atc. In contrast, the addition of atc to the control strain did not affect the amount of cell wall-associated MspA significantly. These experiments, therefore, suggest that the transport of MspA across the cytoplasmic membrane is SecA1 dependent. We could not detect intracellular accumulation of MspA as a result of *secA1* silencing, suggesting that *mspA* translation and secretion are coupled or that intracellular MspA is efficiently degraded.

Many genes that are essential for the optimal growth of *M. tuberculosis* on agar plates have recently been identified by genome-wide mutagenesis (24, 37). More than a third of the approximately 600 genes postulated to be essential for the optimal growth of *M. tuberculosis* are of unknown function. By contrast, only 4% of all essential *B. subtilis* genes are of unknown function (21), suggesting that significant gaps exist in our understanding of essential gene functions in mycobacteria. Functional orthologues for 78% of essential *M. tuberculosis* genes have been retained in the genome of *M. leprae*, even though its genome has undergone extensive degeneration and contains functional orthologues of only 40% of all *M. tuberculosis* genes (8, 37). This suggests that most essential genes are conserved among all mycobacteria. The fast-growing, nonpathogenic *M. smegmatis* might therefore be a valuable surrogate host for the analysis of essential mycobacterial gene functions. We developed a gene expression system that allows the efficient silencing of an essential gene by the addition of atc to the culture of *M. smegmatis*. We further demonstrated that conditional gene silencing allows the identification of candidate substrates of the general secretion pathway in mycobacteria. This suggests that a combination of dose-responsive silencing of essential genes with genomics approaches, for example, transcriptome and proteome analyses, could be used to increase our understanding of protein secretion and other processes essential for the growth of mycobacteria.

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